

Briefly, one to five parasites, obtained by limiting dilution, were directly emerged in the RT-PCR buffer (Stratagene) with different concentration of primers,  $MgCl_2$  KCl and Tris-Cl. Both DNA and RNA were released from the parasites by heating at 93°C for 3 min. The DNA was degraded by addition of 10 U DNase (Stratagene). Reverse transcription was carried out immediately after the addition of random primers and reverse transcriptase (Perkin-Elmer). The PCR reaction was subsequently performed in the same tube. Through comparison of the amplification efficiency from different reactions, the optimised parameters for single cell RT-PCR were found to be as follows: 100mM Tris-Cl, pH 8.3, 35 mM  $MgCl_2$ , 500mM KCl, and the final concentration of primers was 1 $\mu$ M. In the subsequent experiments, individual trophozoite-infected rosetting erythrocytes were isolated with a 5 $\mu$ M glass-pipette using an inverted microscope. The selected pRBC was stripped of uninfected RBC and repeatedly grabbed, ejected and turned to conclusively ensure that it had pigment and that the selected cell was a single trophozoite-infected RBC (see Fig. 1A). Fifty cycles of amplification at 93°C for 20 seconds, 55°C for 30 seconds and 72°C for 1 min were needed for product detection. Several controls were included in each experiment; one blank control (without parasite(s)) and one without reverse-transcriptase to rule out the possibility of contamination and amplification due to the presence of genomic DNA.--

Page 32, replace the paragraph, beginning on line 24, bridging pages 32 and 33, as follows:

EA --A specific upstream primer (L-6, 5'-GAC ATG CAG CAA GGA GCT TGA TAA -3') (SEQ ID NO: 4) in the 434-bp sequence and a downstream primer (L-5, 5'-CCA TCT CTT CAT ATT CAC TTT CTG A -3') (SEQ ID NO: 5) mapping to the conserved sequence of ATS were generated and reverse transcription was carried out as described above. PCR was performed with the Expand<sup>TM</sup> High Fidelity PCR System (Boehringer Mannheim). A single 4.9-kb fragment was amplified, which was digested into three fragments with Hind III and EcoR V and cloned into the pZErO-1 vector (Zero Background, Invitrogen). The sequencing was performed with LongRanger<sup>TM</sup> gel (FMC) on an A.L.F. Sequencer (Pharmacia). The 5' region of the FCR3S1.2-var1 transcript was cloned by screening a cDNA library (Schlichtherle, unpublished) with the 434-bp fragment as probe and seven overlapping fragments were sequenced. The 3' terminal region was cloned by nested RT-PCR. Reverse transcription was primed with oligo-dT and PCR was performed with a specific 5' primer (P-1, 5'-CTT TCG ACT CTA CCA TCC T-3') (SEQ ID NO: 6) upstream of TM region and a 3' primer (P-4, 5'-TTA GAT ATT CCA TAT ATC TGA TA-3') (SEQ ID NO: 7) mapping to the C-terminal sequence of FCR3 (var 2) PfEMP 1. Five overlapping fragments were sequenced. Fourteen overlapping clones were in total sequenced in both directions in order to ensure that the sequence was correct and was transcribed from a single gene.--

Page 34, replace the paragraph, beginning on line 1, as follows:

ES

--Both DBL-1 and ATS fragments were amplified by specific primers (Ex-1.1, 5'-ATC GAA TTC TGC AAA AAA GAT GGA AAA GGA A-3' (SEQ ID NO: 8) and D-1, 5'-GTA TTT TTT TTG TTT GTC AAA TTG-3' (SEQ ID NO: 9) for DBL-1; Ex-2, 5' - ATC GAA TTC TCT GAA AAT TTA TTC CAA A-3' (SEQ ID NO: 10) and P-4 for ATS). The amplified fragments were inserted into the EcoR I cloning site of pGEX-4T-1 downstream of the glutathione S-transferase sequence. The *E.coli* BL21 was used as the expression strain. Expression of both fusion proteins was induced with 0.1 mM IPTG at 30°C for 4h and the fusion proteins were purified on glutathione sepharose (Pharmacia) as described in the instructions provided by the manufacturer (GST Gene Fusion System, Pharmacia). The expression constructs were sequenced by cycle sequencing to check that the recombinant plasmids were of the expected sequences in the correct reading frames. Thrombin cleavage of the fusion proteins was performed according to a standard procedure. Western-blot analysis of DBL-1-GST and ATS-GST fusion proteins was with a biotin labelled anti-GST mAb (clone GST-2, IgG2b, Sigma) and ALP-avidin (Sigma) to reveal the pattern of protein expression. Although the induction of expression was at a low temperature and the purification was in the presence of a cocktail of enzyme inhibitors (0.5mM EDTA, 1mM PEFABLOC (serine protease inhibitor) (AEBSF) Boehringer Mannheim), there was still some breakdown of

E3 the DBL-1-GST. The fusion proteins, stained by the anti-GST mAb, decreased with thrombin treatment. This information together with the knowledge that the plasmids were of the expected sequences ensured that the fusion proteins indeed were the corrected ones.--

Page 35, replace the paragraph, beginning on line 9, as follows:

E4 --Ten-well immunofluorescence glass-slides were precoated with 10% poly-L-lysine in PBS for 30 min. Monolayers of RBC were made by addition of 20 µl of 0.5% 3x washed bloodgroup O Rh<sup>+</sup>RBC in PBS to each well. Twenty µl DBL-1-GST, ATS-GST or GST alone (80µg/ml) in PBS was added to the wells for 30 min. The DBL-1-GST fusion protein was in subsequent experiments incubated in the presence of heparin, heparan sulfate or chondroitin sulfate (titrated from 20 to 8 mg/ml) to study the inhibitory activity of each GAG. Slides were washed 3 times with PBS and the fusion protein-binding was detected with the biotin-labelled anti-GST mAb and an EXTRAVIDIN (a modified avidin) FITC conjugate (Sigma). The fluorescence was assessed in a Nikon Optiphot-2 UV microscope, using a x10 ocular and an oil lens with a magnification of x100.--

IN THE CLAIMS:

Amend claim 13 as follows:

E5 --13. (thrice amended) An isolated polypeptide originating from a malaria erythrocyte membrane protein